

1   **Development of a Liquid Chromatography High Resolution Mass Spectrometry (LC-**  
2   **HRMS) Method for the Quantitation of Viral Envelope Glycoprotein in Ebola Virus-Like**  
3   **Particle Vaccine Preparations.**

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31    **Abstract:**

32    **Background:** Ebola virus like particles; (EBOV VLPs, eVLPs), are produced by expressing the  
33    viral transmembrane glycoprotein (GP) and the structural matrix protein VP40 in mammalian  
34    cells. When expressed, these proteins self-assemble and bud from 'host' cells displaying  
35    morphology similar to infectious virions. Several studies have shown that rodents and non-  
36    human primates vaccinated with eVLPs are protected from lethal EBOV challenge. The mucin-  
37    like domain of envelope glycoprotein (GP<sub>1</sub>) serves as the major target for a productive humoral  
38    immune response. Therefore GP<sub>1</sub> concentration is a critical quality attribute of EBOV vaccines  
39    and accurate measurement of the amount of GP<sub>1</sub> present in eVLP lots is crucial to understanding  
40    variability in vaccine efficacy.

41    **Methods:** After production, eVLP's are characterized by determining total protein concentration  
42    and by western blotting, which only provides semi-quantitative information for GP<sub>1</sub>. Therefore, a  
43    liquid chromatography high resolution mass spectrometry (LC-HRMS) approach for accurately  
44    measuring the concentration of GP<sub>1</sub> in eVLP's was developed. The method employs isotope  
45    dilution mass spectrometry using 4 target peptides from 2 regions of the GP<sub>1</sub> protein. Purified  
46    recombinant GP<sub>1</sub> was generated to serve as an assay standard. GP<sub>1</sub> quantitation in 5 eVLP lots  
47    was performed on an LTQ-Orbitrap Elite MS and the final quantitation was derived by  
48    comparing the relative response of the 200 fmol AQUA peptide standards to the analyte response  
49    at 4 ppm.

50    **Results:** Conditions were optimized to ensure complete tryptic digestion of eVLP, however,  
51    persistent missed cleavages were observed in the target peptides. Additionally, N-terminal  
52    truncated forms of the GP<sub>1</sub> protein were observed in all eVLP lots, making peptide selection  
53    crucial. The LC-HRMS strategy resulted in the quantitation of GP<sub>1</sub> with a lower limit of

54 quantitation (LLQ) of 1fmol and an average percent coefficient of variation (CV) of 7.4 %.

55 Unlike western blot values, the LC-HRMS quantitation of GP<sub>1</sub> in 5 eVLP vaccine lots correlated

56 positively with survival (after EBOV challenge) in mice.

57 **Conclusions:** This method provides a means to rapidly determine eVLP batch quality based

58 upon the quantitation of antigenic GP<sub>1</sub>. By monitoring variability based on GP<sub>1</sub> content, the

59 eVLP production process can be optimized, and the total amount of GP<sub>1</sub> needed to confer

60 protection can be accurately determined.

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64 **Keywords:**

65 Ebola virus, virus like particles, high resolution mass spectrometry, stable isotope dilution

66 quantitation.

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92 **Background:**

93       Ebola is an extremely pathogenic virus that causes hemorrhagic fever and can result in  
94       mortality rates of up to 90%. The 2014 Ebola endemic in West Africa brought global attention  
95       to a disease that was once only an isolated-regional problem. More than a year later and with a  
96       death toll greater than 10,000 people, there is an urgent need for novel therapeutic strategies  
97       including treatment and prevention. Virus-like-particles (VLPs) represent a new type of  
98       prophylactic vaccine that has had success and is commercialized in products such as, Cervarix  
99       (human papillomavirus) [1], and Gardasil (human papillomavirus) [2]. Virus-like particles  
100      (VLPs) are generated by exploiting the intrinsic ability of structural viral component proteins,  
101      frequently major proteins in the capsid or envelop, to spontaneously self-assemble when  
102      expressed in mammalian cells [3]. VLPs are therefore composed of a subset of viral components  
103      that mimic the wild-type virus structure but lack viral genetic material, rendering them non-  
104      infectious. Unlike recombinant protein vaccines which may elicit a weak immune response due  
105      to non-ideal presentation of the viral antigens to the immune system, VLPs are usually  
106      antigenically indistinguishable from infectious virus particles [4-6]. These properties make VLPs  
107      promising candidates for new efficacious vaccines against many viral pathogens including  
108      filoviruses such as Ebola.

109       Ebola Virus (EBOV) VLPs (eVLPs) are produced by transfection of HEK293 cells with  
110      plasmids encoding the genes for viral matrix protein VP40, and envelope glycoprotein (GP) [7-  
111      9]. The envelope GP is solely responsible for viral attachment, fusion, and entry of new host  
112      cells, and it is therefore a major target of vaccine design efforts. When these proteins are  
113      expressed in mammalian cells, they self-assemble and bud from lipid rafts resulting in eVLPs  
114      that contain GP, VP40, and other packaged host proteins [10].

115        Each of the seven genes which comprise the EBOV genome is transcribed into individual  
116    messenger RNAs (mRNAs) with the exception of the fourth gene, which encodes for GP. In  
117    virus-infected cells, several GP-specific mRNAs are synthesized due to a transcriptional RNA  
118    editing phenomenon. Envelope GP is not the primary product of the fourth gene but instead is  
119    generated through transcriptional editing, which leads to the insertion by the viral polymerase of  
120    an extra adenosine into a stretch of seven other adenosine residues (total 8A) at a specific-editing  
121    site near the middle of the coding region [11]. The EBOV polymerase transcribes the unedited  
122    GP gene which contains 7 adenosines at the editing site most of the time (>80%), and these  
123    transcripts result in the expression of the predominant GP gene product, secretory glycoprotein  
124    (sGP) [12]. The addition of 2 adenosine residues at the editing site (total of 9A) codes for a third  
125    GP gene product known as second secreted GP (ssGP). Both secreted forms have the same  
126    amino-terminal 295 amino acids as GP. Editing (total of 8A) results in the continuation of GP for  
127    381 amino acids beyond the divergence point (see **Figure 1** for sequence alignment) resulting in  
128    the pre-processed GP polypeptide (GP0). GP0 is proteolytically cleaved to a large amino-  
129    terminal fragment (GP<sub>1</sub>) and a smaller carboxy-terminal fragment (GP<sub>2</sub>) in the trans-Golgi  
130    network by a furin-like enzyme [13]. Mature envelope GP is formed by the re-association of GP<sub>1</sub>  
131    and GP<sub>2</sub> through disulfide bonding, and the GP<sub>1,2</sub> complex is anchored in the membrane by a  
132    transmembrane domain near the carboxy terminus of GP<sub>2</sub> [14, 15]. GP<sub>1</sub> contains a highly  
133    glycosylated mucin-like domain (MLD) and antibodies that recognize this region of GP<sub>1</sub> have  
134    been shown to be protective in mouse models of lethal Ebola virus challenge [16]. In addition,  
135    many neutralizing antibodies, including two that comprise part of a promising therapeutic  
136    cocktail [17], are directed against the MLD [16, 18, 19].

137        The GP expression vector used to produce eVLP in HEK293 cells encodes for a  
138    transcript containing 8 adenosines and thus should produce only GP<sub>1,2</sub>. Large scale production of  
139    eVLPs is performed by contract manufacturing organizations and each lot is characterized by  
140    assays that measure total protein and GP<sub>1</sub> concentrations (western blotting or single antibody  
141    ELISA). Ongoing vaccine studies in our laboratory have shown that eVLPs provide protection  
142    against a lethal dose of EBOV in mice and non-human primates when administered with an  
143    appropriate adjuvant [20, 21]. Vaccine dosages are administered based on GP<sub>1</sub> protein  
144    concentration; however, the effectiveness (based on survival) of each small scale VLP  
145    preparation can be highly variable. Therefore improved methods are needed to serve as lot  
146    release assays for each eVLP preparation to ensure that only material of sufficient quality is used  
147    for *in vivo* evaluation.

148        This report describes the development of an isotope dilution LC-HRMS method for the  
149    absolute quantitation of Ebola GP<sub>1</sub> in eVLP. This protocol resulted in the quantitation of GP<sub>1</sub>  
150    with a limit of quantitation of 1fmol and an average percent coefficient of variation of 7.4 %.  
151    The optimized MS quantitation of GP<sub>1</sub>, in contrast to the western blot quantitation correlated  
152    with survival in vaccinated mice after EBOV challenge. This assay provides a means to monitor  
153    eVLP batch variability based on GP<sub>1</sub> content, provides a means of monitoring product purity  
154    during process development, and will assist in the determination of the dosage needed to confer  
155    protection in vaccinated animals.

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157    **Materials and Methods:**

158    ***Generation and Characterization of eVLPs.***

159 eVLPs were produced under a contract with Paragon Bioservices (Baltimore, MD) using a  
160 modification of the procedure described by Warfield et al. [22]. In brief, eVLPs were created by  
161 transfecting HEK 293 cells with expression vectors containing the genes for GP and VP40  
162 proteins [7, 22-24]. To purify the eVLPs, the clarified cell supernatants were pelleted, separated  
163 on a 20–60% continuous sucrose gradient, concentrated by a second centrifugation, and  
164 resuspended in endotoxin-free PBS. The gradient fractions containing the eVLPs were  
165 determined using western blotting using an anti-GP<sub>1</sub> antibody (6D8). The total protein  
166 concentration of each eVLP preparation was determined in the presence of Nonidet P-40  
167 detergent using a detergent-compatible protein assay (Bio-Rad). For these blots unpurified  
168 recombinant GP material was used as an assay standard for the generation of a standard curve.

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170 ***Generation and Characterization of a Recombinant GP<sub>1</sub> standard.***

171 A batch of recombinant Ebola glycoprotein (rGP, carrying an N-terminal poly-histidine tag) was  
172 expressed in human HEK293 cells and subsequently purified by immobilized metal affinity  
173 chromatography (IMAC). The material was produced under a contract with the Frederick  
174 National Laboratory for Cancer Research (Frederick, MD). Analytical scale reverse phase  
175 chromatography was used to further fractionate the protein preparation under reducing  
176 conditions. Recombinant Ebola glycoprotein material was reduced with 2-mercaptoethanol (final  
177 concentration, 0.5 M) during a 30 minute room temperature incubation and then injected (300 µg  
178 total protein) onto an apHera C4 column (150 mm x 4.6 mm, 5 µm; Supelco). Mobile phases  
179 were as follows: (A) 0.1% trifluoracetic acid (TFA) and (B) acetonitrile/0.1% TFA. The flow  
180 rate was set to 0.5mL/min and rGP was separated using the following gradient: 0-3 min: 10% B,  
181 3-5 mins: 10 to 20% B, 5-65 mins: 20 to 45% B, 65-71 mins: 45 to 80% B, and 72-82 mins: 80

182 to 10% B. During the 20-45% B gradient, nine peaks were collected and dried to completion in a  
183 vacuum concentrator. All GP<sub>1</sub> purification experiments were conducted using an Agilent 1200  
184 HPLC system equipped with a UV detector; eluents were continuously monitored at 214 nm.

185 Each fraction of purified rGP<sub>1</sub> was re-dissolved in 100  $\mu$ l of 8M urea/PBS. The protein  
186 concentration of each fraction was estimated by measuring the optical density (OD) at 280 nm in  
187 a spectrophotometer and assuming an extinction coefficient at 1% equal to 10 (under this  
188 assumption, a 1 mg/ml solution of a protein would have an OD reading of 1.0). Protein from  
189 each fraction (500 ng) and 1  $\mu$ g of the original unfractionated GP material were resolved on a 4-  
190 12% BOLT SDS PAGE gel (Life Technologies) and stained with silver (Pierce Silver Stain kit,  
191 Fisher Scientific) following the manufacturer's instructions. Following the initial  
192 characterization experiment, a larger scale purification experiment was conducted to obtain a  
193 sufficient quantity of GP<sub>1</sub>. In this iteration, 300  $\mu$ g of unpurified recombinant GP material was  
194 fractionated by reverse phase HPLC and a single peak corresponding to GP<sub>1</sub> was manually  
195 collected. The OD at 280 nm was recorded and a preliminary protein concentration was  
196 determined for the sample using a theoretical molar extinction coefficient of 45,380 (calculated  
197 from the primary sequence of GP<sub>1</sub> using the protein parameter tool on the ExPASy server,  
198 <http://web.expasy.org/protparam/>). The sample was subsequently aliquoted and dried under  
199 vacuum centrifugation. SDS PAGE was used to compare the rGP<sub>1</sub> pool to the original  
200 unfractionated rGP material. For this experiment, 2.5  $\mu$ g of rGP<sub>1</sub> and 3.3  $\mu$ g of unfractionated  
201 rGP were resolved on a 4-12% BOLT SDS PAGE gel (Life Technologies) and stained with  
202 Coomassie Blue (Imperial protein stain, Fisher Scientific). Lastly, the protein content of the  
203 pooled and purified rGP<sub>1</sub> preparation was determined by amino acid analysis (AAA) following

204 acid catalyzed hydrolysis by Biosynthesis (Lewisville, TX). AAA conducted on triplicate rGP<sub>1</sub>  
205 samples determined that on average, each aliquot contains 1.8 µg of protein.

206

207 ***Western Blot Analysis.***

208 Based on total protein concentration, approximately 20-50ng of each eVLP lot was loaded onto a  
209 4-12% SDS PAGE gel and run under reducing conditions. Known amounts of recombinant  
210 Ebola GP material (purified GP<sub>1</sub> and unpurified) were also loaded on the gel. Two separate gels  
211 were run for the eVLP lots tested and transferred to PVDF membranes. Each blot was blocked  
212 overnight with Odyssey blocking buffer in phosphate buffered saline (PBS) (LI-COR  
213 Biosciences Lincoln, NE) and then incubated with primary antibody against GP<sub>1</sub> (6D8 or  
214 F88.H3D5, 1:1000) for 1 hour at room temperature. After washing 3X with PBS + 0.1% Tween-  
215 20 for 5 minutes, secondary antibody (1:5000) goat  $\alpha$ -mouse IRDye® 680 labelled (LI-COR)  
216 was added and the blots were incubated an additional hour. The blots were again washed 3X  
217 with PBST, and then stored in PBS until visualized with an Odyssey infrared imaging system  
218 (LI-COR Biosciences Lincoln, NE: model number 9210).

219

220 ***Preparation of eVLP and rGP<sub>1</sub> Standard Proteolytic Digests***

221 Upon receipt of each lot of eVLP from the contractor, stocks were divided into 10 µg aliquots  
222 based on the total protein concentration and stored at -80°C until use. For simplicity, each of the  
223 5 lots of eVLP used in this study was designated using alphabetical values (A-E). Sample  
224 preparation for MS was performed by first increasing the volume of each aliquot to 50µL with  
225 'Solution tA' (25mM Tris-HCl, pH 8.0), reducing with 55 mM DTT at 55°C for 30 minutes, and  
226 then alkylating with 68 mM iodoacetamide at room temperature for 45 minutes. Both of these

227 steps were performed in the presence of 0.05% ProteaseMax™ (Promega Madison, WI). The  
228 total volume was then increased to 95  $\mu$ L with ‘Solution tD’ (25 mM Tris-HCl, pH 8.0, 10%  
229 acetonitrile) and 4  $\mu$ L of a 0.1  $\mu$ g/ $\mu$ L sequencing grade trypsin/lys-C solution (Promega) and 1  
230  $\mu$ L of 1% ProteaseMax™ were added followed by incubation at 42°C for 4 hours. Digests were  
231 heated to 90°C for 5 minutes, dried completely by speed-vac and stored at -80°C until analyzed.  
232 The purified rGP<sub>1</sub> standard was digested using the same protocol as the eVLP’s with the  
233 exception that the concentration of the trypsin/lys-C was reduced 4-fold.

234

235 ***Quantitation of GP<sub>1</sub> by LC-HRMS***

236 AQUA Ultimate™ peptides (Thermo Fisher Scientific) were synthesized based on the results of  
237 extensive survey runs of digested and purified rGP<sub>1</sub> to determine which endogenous peptide  
238 sequences had the fewest possible post-translational or artefactual modifications and that also  
239 resulted in unambiguous MS<sup>2</sup> spectra for identification, as well as a consistent and  
240 chromatographically distinct extracted ion chromatograms (XIC) for quantitative measurement.  
241 The following four peptide sequences were selected: 301-IRSEELSFTA VSNR-314, 303-  
242 SEELSFTA VSNR-314, 65-SVGLNLEGNGVATDVPSATK-84, and 65-  
243 SVGLNLEGNGVATDVPSATKR-85. Each peptide had a C-terminal amino acid modified with  
244 <sup>13</sup>C and <sup>15</sup>N isotopes resulting in a 10 Da and 8 Da mass increase for arginine and lysine  
245 respectively. AQUA peptides were supplied by the manufacturer in a 40% acetonitrile, 0.1%  
246 formic acid solution at 5pmol/ $\mu$ L. A 2X working solution was prepared in 40% acetonitrile,  
247 0.1% formic acid by adding 8  $\mu$ L of each stock peptide into a total volume of 200  $\mu$ L (200  
248 fmol/ $\mu$ L). The analyte digest was resuspended in 60 $\mu$ L or 80  $\mu$ L 40% acetonitrile, 0.1% formic  
249 and a 5-point, 2-fold serial dilution performed. AQUA peptides were then spiked into each

250 analyte dilution at a 1:1 (v:v) ratio resulting in a 100 fmol/µL AQUA standard concentration. In  
251 addition, a blank was prepared by diluting the AQUA standards 1:1 with 40% acetonitrile, 0.1%  
252 formic acid. Samples were resolved on an Acclaim PepMap 100 column (1mm x 100mm)  
253 packed with 3um, 100A C18 particles and analyzed in triplicate from lowest to highest  
254 concentration by loading 2 µL onto an Ultimate 3000 HPLC (Thermo Fisher Scientific), Mobile  
255 phases were as follows: (A) 0.1% formic acid (FA) and (B) acetonitrile/0.1% FA. The flow rate  
256 was set to 75 µL/min and peptides were eluted using a 17-minute linear gradient of 1-34%  
257 mobile phase B. The column eluent was connected to an Orbitrap Elite mass spectrometer with a  
258 HESI-2 ion source (Thermo Fisher Scientific) with a sheath gas pressure of 20 psi and an  
259 auxiliary gas flow of 5 units. The electrospray ionization voltage was 5.0 kV with an ion transfer  
260 tube temperature of 350 °C and S-lens RF at 50%. The automatic gain control target was 5.0  
261 x10<sup>4</sup> for Orbitrap in SIM mode and 1.0 x10<sup>4</sup> for linear ion trap in MS/MS mode. The maximum  
262 injection time for MS/MS was set to 30 milliseconds. Four consecutive 200 amu SIM scans over  
263 the range of m/z 415-1215 at a resolution of 60,000 were used to detect the 20 ions of interest  
264 followed by 4 targeted MS/MS low resolution CID scans of the most prominent analyte peptides  
265 for sequence verification. For each peptide (heavy and light), both the doubly and triply charged  
266 ions were considered and used for quantitation. The average of triplicate extracted ion  
267 chromatogram (XIC) counts of each of the 4 standard AQUA peptides, the 4 analyte peptides  
268 and deamidated SVG peptides were obtained using XCalibur 2.0 (Thermo Scientific) with  
269 automatic integration baseline window set at 10 scans, area noise factor at 5, and peak noise  
270 factor set to 20. The XIC counts from each SVG, SVGR, SVG<sup>deam</sup>, and SVGR<sup>deam</sup> peptide  
271 charge state were first summed in each individual replicate run and then the average for the three  
272 technical repeats was determined to represent the contribution of Peptide Set 2 at each dilution.

273 The SEE and IRSEE (Peptide Set 1) values were obtained similarly. The AQUA standard  
274 peptide XIC counts were then used to calculate the ratio of AQUA peptide standard to the 'light'  
275 analyte peptide at each dilution using a mass tolerance of 4 ppm. This ratio or relative response  
276 was used to generate standard curves which were then used to determine the amount of analyte in  
277 fmols injected on-column. These fmol values were then converted to  $\mu\text{g}$  to calculate the total  
278 GP<sub>1</sub> using a total protein mass of 54,768 Da (UniProt entry Q05320, 33-501).

279

280 ***Limit of Quantitation and Linearity of Analyte Peptides***

281 A previously quantified digest of a Brown Lot eVLP was diluted to 140 fmol/ $\mu\text{L}$  GP<sub>1</sub> in 40%  
282 acetonitrile, 0.1% formic acid and serially diluted 2-fold down to 0.5 fmol/ $\mu\text{L}$  for a total of 9  
283 dilutions. Using a 2  $\mu\text{L}$  injection volume, each dilution was run in triplicate as described above  
284 and XIC area standard curves generated for each of the 4 quantitation peptides ranging from 275  
285 fmol to 1.0 fmol. The similar procedure was carried out on the AQUA peptides except the  
286 dilution was carried to 0.4 fmol/ $\mu\text{L}$ .

287

288 ***Deamidation of AQUA Peptide Standards***

289 A 40 pmol aliquot of AQUA SVG peptide was resuspended in 200  $\mu\text{L}$  50 mM NH<sub>2</sub>HCO<sub>3</sub> pH 8.1  
290 and incubated at 50°C for 3 days then dried to completion by speed-vac. The sample was  
291 resuspended in 200  $\mu\text{L}$  40% acetonitrile, 0.1% formic acid and 2 $\mu\text{L}$  was injected using the  
292 instrument and chromatographic conditions outlined above. Target masses were aligned by  
293 charge state and retention time and XIC values derived as described above using a mass  
294 tolerance of 4 ppm.

295

296 ***In-Gel Trypsin Digestion***

297 A 5  $\mu$ g aliquot of VLP was fractionated by SDS-PAGE onto a 4%-12% gel (BioRad) and the 10  
298 highest intensity bands excised and minced into 1 x 1 mm plugs. Each sample was serially  
299 processed in 100  $\mu$ L solution **tA**, then solution **tB** (25mM Tris-Cl, pH 8.0, 50% Acetonitrile),  
300 and finally 100% Acetonitrile before being evaporated to dryness in a SpeedVac. Each gel slice  
301 was then reduced and alkylated by incubation in 55 mM DTT at 55°C followed by incubation  
302 with 68 mM Iodoacetamide for 45 minutes at room temperature. Bands were dried to  
303 completion and 10  $\mu$ L of a 12.5 ng/ $\mu$ L sequencing grade modified trypsin solution (Promega,  
304 Madison, WI) in solution **tD** was added and incubated at room temperature for 30 minutes until  
305 trypsin was absorbed. 70  $\mu$ L solution **tD** was then added and samples incubated overnight at  
306 37°C. Peptides were then extracted 2X by incubating in 50% Acetonitrile, 0.1% formic acid and  
307 the combined digest dried to completion by speedvac.

308

309 ***Animals, Vaccinations, and Viral challenge***

310 Research was conducted under an IACUC approved protocol in compliance with the Animal  
311 Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and  
312 experiments involving animals. The facility where this research was conducted is accredited by  
313 the Association for Assessment and Accreditation of Laboratory Animal Care, and adheres to  
314 principles stated in the Guide for the Care and Use of Laboratory Animals, National Research  
315 Council, 2011. C57BL/6 mice were obtained from NCI Charles River. Female mice between 8-  
316 12 weeks of age were vaccinated with 100  $\mu$ l injections containing 10  $\mu$ g of GP (as determined  
317 by western blot) via the intramuscular (IM) route, in the caudal thigh. Each lot of eVLP was  
318 irradiated at 1e6 rad to ensure sterility and contained less than 25 EU/ml endotoxin and less than

319 10 colony forming units (CFU) of bacteria per vaccination. VLP were diluted in sterile saline and  
320 vaccinations were administered two times, with three weeks between vaccinations. Viral  
321 challenge occurred four weeks after the second vaccination. A challenge dose of 1,000 pfu of  
322 mouse-adapted Ebola virus [25] was administered via the intraperitoneal route (IP). The survival  
323 data was pooled from 2-3 studies with n=10 mice each.

324

325 ***Statistical Analysis (differences between lots, animal survival rates.)***

326 Survival studies were evaluated using Fisher's exact test with multiple testing corrections  
327 performed by permutation based on the number of comparison's performed. The significance of  
328 the deviation from a null hypothesis (p value) was reported for the survival observed in animals  
329 vaccinated with each eVLP lot.

330

331 **Results:**

332 **Selection and Evaluation of GP<sub>1</sub> Target Peptides for Quantitation by LC-HRMS**

333 In the development of a reproducible protein MS quantitation scheme, the selection of target  
334 peptides is a crucial step, especially when the protein of interest is expressed in multiple  
335 isoforms, and is highly post-translationally modified. In both the infectious virions and eVLP  
336 preparations, GP<sub>1</sub> and GP<sub>2</sub> are proteolytically processed from the GP0 polypeptide and disulfide  
337 linked to form the mature GP<sub>1,2</sub> transmembrane protein complex [2, 15] (see **Figure 1A**). Four  
338 peptides were initially identified as target candidates for the quantitation of GP<sub>1</sub> primarily due to  
339 their ionization characteristics, lack of post-translational modifications and relative distance  
340 within the sequence. During initial LC-HRMS method development we found two of these  
341 peptides (173- *GTTFAEGVVAFLILPQAK*[<sup>13</sup>C6, <sup>15</sup>N2]-190) and (479-

342 *LGLITNTIAGVAGLITGGR*[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub>]-497) failed to show consistent linearity. The GTT  
343 peptide and LGL peptide have a Grand Average of Hydropathy score (GRAVY) [26] of 0.933  
344 and 1.08 respectively, indicating a high level of hydrophobicity, which can hinder reliable  
345 quantitation. The remaining 2 peptides 65-*SVGLNLEGNGVATDVPSATK*[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>2</sub>]-84 and  
346 303-*SEELSFTAVSNR*[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub>]-314 (designated SVG and SEE, respectively) provided highly  
347 reproducible linear standard curves and were selected for use in the assay (see **Figure 1A and**  
348 **1B**). The selection of these 2 peptides, as we subsequently discovered, also offered a way to  
349 distinguish full length GP<sub>1</sub> from truncated versions of the protein, as the SEE peptide sequence is  
350 found only in the full length GP<sub>1</sub> molecule. Isotopically labelled AQUA Ultimate™ peptides  
351 (Thermo Scientific) of each peptide sequence were synthesized and used for quantitation.  
352 Synthetic AQUA (Absolute QUAntitation) peptides are chemically and physically  
353 indistinguishable from their endogenous counterparts with respect to retention time, ionization  
354 efficiency, and MS/MS fragmentation except they are modified to include <sup>13</sup>C and <sup>15</sup>N isotopes  
355 that increase their relative mass by very precise increments [21]. For this study, AQUA  
356 Ultimate™ peptides (Thermo Fisher Scientific) were selected as they have the highest available  
357 concentration precision and purity.

358 During the initial survey runs which were conducted to optimize the digestion of the  
359 eVLP for completeness and reproducibility, it was observed that two missed cleavage sites  
360 appeared regularly: a C-terminal arginine on the SVG peptide and an N-terminal arginine on the  
361 SEE peptide. We rigorously searched for additional missed cleavages as well as non-specific  
362 cleavages upstream and downstream of the fully tryptic peptides, and we found no evidence that  
363 these species were present (see **Supplemental Table 1A and 1B**). Given that the ratio of missed  
364 cleavage to fully tryptic peptides was highly variable (0.4% - 45%), the 2 peptides representing

365 these missed cleavages (65-SVGLNLEGNGVATDVPSATKR[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub>]-85 and 301-  
366 **IRSEELSFTA**VSNR[<sup>13</sup>C<sub>6</sub>, <sup>15</sup>N<sub>4</sub>]-314) were synthesized and evaluated for reproducibility and  
367 linearity. These peptides were chromatographically distinct, generated linear standard curves  
368 and were therefore suitable for use in the quantitation assay (see **Figure 1B** and **Supplemental**  
369 **Figure 1**). It was also observed that one of the two asparagine residues in the endogenous SVG  
370 peptide, but not both, were routinely deamidated. Since all XIC counts from this species need to  
371 be combined in order to account for the full stoichiometric contribution of the SVG peptide we  
372 evaluated whether the non-deamidated AQUA SVG peptide standard could be used to quantitate  
373 the level of deamidated analyte peptide. The standard AQUA SVG and SVGR peptides were  
374 fully deamidated by incubating them at 55°C for 2.5 days at pH 8.1 (see **Supplemental Figure**  
375 **2**). Interestingly, even with this harsh treatment, the doubly deamidated species comprised only  
376 5% of the total SVG peptide compliment, indicating that under normal processing conditions it  
377 would be a highly unlikely modification. The XIC response of the deamidated peptide standards  
378 were then compared to the non-treated peptide standard of the same concentration. As shown in  
379 **Figure 1C**, the response was essentially identical. Therefore, the XIC counts derived from the  
380 SVG and SVGR standard AQUA peptides can be used to quantify the additional XIC counts  
381 from the endogenous deamidated peptide species without having to synthesize additional labeled  
382 deamidated standards. We did not observe deamidation of the single asparagine in the SEE target  
383 peptide.

384

385 **Determination of Optimal Digestion Conditions for GP<sub>1</sub> within the eVLPs.**

386 The proteolytic enzyme of choice is a mass spectrometry grade Trypsin/Lys-C combination  
387 (Promega V5073) as it is well characterized, versatile and highly specific. Initial digestion

388 experiments and LC-HRMS analysis of the eVLPs revealed that some regions of GP<sub>1</sub> are very  
389 resistant to proteolytic digestion even in the presence of enhancing surfactants such as  
390 ProteaseMax™. To ensure as complete a digestion of the eVLP GP<sub>1</sub>, we conducted extensive  
391 testing using a variety of buffer formulations, reagents, and pre-digestion treatments. These  
392 treatments included deglycosylation, sonication and high temperature. Since GP<sub>1,2</sub> is a heavily  
393 glycosylated membrane embedded protein, we performed PNGase deglycosylation prior to  
394 digestion in the hope of reducing steric hindrance of the sugars and thereby enhancing trypsin  
395 proteolysis. Although we observed a modest improvement in overall peptide count as well as a  
396 reduction in frequency of the SVG/SEE missed cleavages, we did not observe any appreciable  
397 differences in the ratios of the peptides selected for use in quantitation (data not shown). We  
398 concluded the additional deglycosylation procedure would only add to the complexity of the  
399 assay. We also tested the cleavable detergent/surfactant, ProteaseMax™ (Promega, Madison,  
400 WI), which is designed to enhance the performance of trypsin, and is especially useful for  
401 membrane proteins. This reagent dramatically reduced the overall number of missed cleavages  
402 and allowed the digest time to be reduced from 16 hours to 4 hours without any loss of digestion  
403 efficiency. Despite these efforts, we were unable to completely eliminate the occurrence of the  
404 target peptide missed cleavages described above. However, we did not observe any additional  
405 upstream or downstream missed cleavage species from either target peptide in survey runs from  
406 each eVLP lot tested (**Supplemental Table 1**). Missed cleavage species were observed in 8.2%  
407 of the SEE peptide and 31% in the SVG peptide. These values represent the typical level  
408 observed in all 5 eVLP lots tested after trypsin digestion. We therefore concluded that the 4  
409 peptides selected for the assay would be adequate for quantitation of GP<sub>1</sub> present in eVLP

410 preparations. The final peptide sequences and charge states used for quantitation of Ebola GP<sub>1</sub>  
411 are shown in **Table 1**.

412

413 **Reverse Phase Purification of GP<sub>1</sub> Standard**

414 In any protein quantitation experiment, the assumption is that unique peptides from different  
415 regions within a protein would display a 1:1 molar relationship. However, early quantitation  
416 experiments with test lots of recombinant GP material and eVLP revealed a variable target  
417 peptide (SVG:SEE) stoichiometric ratio between the lots which was otherwise consistent within  
418 each lot. In some cases the disparity between the SVG quantitation and the SEE quantitation  
419 was as high as 25%. In order to rule out experimental error as the cause of the discrepancy, we  
420 prepared a pure monomeric full length rGP<sub>1</sub> standard from recombinant GP material that could  
421 be used to assess the accuracy of the quantitation method. As shown in **Figure 2A**, a reverse  
422 phase chromatography procedure was performed that fractionated reduced rGP material into  
423 multiple sub-species. Fractions were collected and processed for SDS PAGE analysis and silver  
424 stained. As seen in **Figure 2B**, fractions 1-4 and fractions 6-7 constitute GP<sub>1</sub> and GP<sub>2</sub>,  
425 respectively. Interestingly, fractions 1-4 yielded nearly identical SDS PAGE profiles despite  
426 observing multiple shoulder peaks on the reverse phase chromatogram. Ultimately however, the  
427 fractionation procedure resulted in a significant enrichment of individual protein species within  
428 the rGP preparation. As seen in **Figure 2C**, SDS PAGE analysis confirmed that the fractionated  
429 material was highly enriched for GP<sub>1</sub>. Collectively, this data indicates that the procedure  
430 significantly reduced the amount of heterogeneity in the original sample and produced an  
431 enriched version of GP<sub>1</sub> that was suitable for use as an assay standard.

432

**433 Validation of the Quantitation Method with Purified rGP<sub>1</sub> Standard**

434 Quantitative AAA analysis indicated each aliquot of purified rGP<sub>1</sub> contained an average of 1.8  
435 µg GP<sub>1</sub> protein. In order to evaluate the accuracy of our method, four rGP<sub>1</sub> aliquots were  
436 resuspended in either 60 µL or 80 µL 40% acetonitrile, 0.1% formic acid and quantitated using  
437 the newly developed HRAM LC-MS/MS. As seen in **Table 2**, after averaging the individual  
438 peptide set values, the GP<sub>1</sub> concentration was determined to be 1.50 µg/aliquot for trial 1 and  
439 1.49 µg/aliquot for trial 2 using a dilution volume of 60 µL. These values are within 16.8% and  
440 17.4% of the value obtained with AAA (1.8µg). The SVG/SEE stoichiometric disparity,  
441 designated as  $\Delta S$ , was -7.00% for trial 1 and 9.3% for trial 2. For trials 3 and 4 using a dilution  
442 volume of 80 µL, the GP<sub>1</sub> concentration was 1.56 and 1.64 µg/aliquot respectively. These values  
443 are within 13.4% for trial 3 and 9.9% for trial 4 of the value determined by AAA analysis. These  
444 data indicate that the LC-HRMS method and the combination of these 4 peptides (Set 1 and Set  
445 2) is sufficient to account for the GP<sub>1</sub> protein present with an average accuracy 85.6%.

446

**447 Development of a High Resolution/Accurate Mass (HR/AM) Quantification of GP in  
448 eVLPs:**

449 Since the purified rGP<sub>1</sub> standard returned acceptable LC-HRMS quantitation results with both  
450 target peptide sets, we sought to determine the disparity observed in the quantitation of GP<sub>1</sub> in  
451 the eVLPs when using Set 1 and Set 2 peptide pairs. While the eVLPs are designed to produce  
452 only GP<sub>1,2</sub> by altering the primary sequence used to transfect the HEK293 cells, the presence of  
453 multiple forms of GP was observed by western blotting using two monoclonal antibodies with  
454 epitopes located in different portions of the molecule (see **Figure 2D&E**). The mouse  
455 monoclonal antibody 6D8 binds at amino acids 389-405 and therefore has affinity for only Ebola

456 GP<sub>1</sub> [16]. This is the antibody routinely employed for the determination of GP content in the  
457 eVLP preparations by quantitative western blot or ELISA. Antibody H3D5 is a mouse  
458 monoclonal antibody which binds at amino acids 72-109 and therefore has affinity for all forms  
459 of GP (both secreted and membrane bound) (see **Figure 1A**) each containing the SVG peptide  
460 sequence. This antibody has reactivity with all subtypes of Ebola GP<sub>1</sub>, for all subspecies [27]. As  
461 shown in **Figure 2D** and **2E**, the predominant band visualized in the unfractionated rGP material,  
462 purified rGP<sub>1</sub>, and 2 lots of eVLPs (lots 'A' and 'E') using both antibodies is fully glycosylated  
463 GP<sub>1</sub> (~140kDa), however the H3D5 blot shows the presence of strong distinct bands of a lower  
464 molecular weight (~50-100 kDa) present in both eVLP lots and the unpurified rGP material.  
465 These bands are much reduced in the rGP<sub>1</sub> purified standard. The additional bands visible in the  
466 eVLP western blot using the H3D5 antibody do not correspond to the correct molecular weight  
467 for either sGP or ssGP (50 and 47 kDa respectively). In order to verify sequence identity these  
468 bands were excised from a gel of one eVLP lot ('A') and stained for total protein with coomassie  
469 blue. The 10 most intense bands were excised, trypsin digested, and analyzed with long-gradient  
470 CID survey runs as well as targeted LC-HRMS MS to identify any GP protein fragments  
471 contributing to the peptide quantitative variability. The results of this sequencing experiment are  
472 shown in **Supplemental Figure 3**. All bands excised were confirmed to contain EBOV GP<sub>1</sub> or  
473 GP<sub>2</sub> peptides. A gradual loss of C-terminal peptide identifications for GP<sub>1</sub> was observed as the  
474 smaller products visible in the gel were sequenced, suggesting the presence of truncated forms of  
475 GP<sub>1</sub> in the eVLP. This data indicates that peptides derived from the N-terminus would not be  
476 suitable candidates for quantitation of GP<sub>1</sub>. We therefore concluded that the SEE target peptide  
477 (Set 1) was the only reliable standard for the quantitation of GP<sub>1</sub> in eVLP.

478

**479 Testing of the LC-HRMS Quantitation Method for Reproducibility with an eVLP Digest**

480 Three aliquots from a single test lot of eVLP (lot 'A') were used to evaluate the quantitation  
481 method for reproducibility. As shown in **Figure 3A**, the workflow was as follows: heavy AQUA  
482 standard peptides were added at a fixed concentration of 200 fmols/injection while varying the  
483 concentration of the eVLP analyte digest (10  $\mu$ g based on total protein concentration) over 4  
484 two-fold dilutions. Each complete quantitation set contained a 200 fmol/injection AQUA  
485 peptide standard blank and was run in triplicate from which average XIC and percent CV values  
486 were calculated. The XIC area contributions from each charge state were summed to provide the  
487 total fmols for each peptide species (see **Table 3**). The entire quantitation was performed in  
488 triplicate with an analyte resuspension volume of 120 $\mu$ L. Replicates 1, 2, and 3 resulted in a  
489 calculated GP<sub>1</sub> concentration (based on the SEE peptide standard set 1 only) of 0.62, 0.53 and  
490 0.61 mg/ml, respectively with an average of 0.59 mg/ml  $\pm$  0.025 mg/ml and a percent CV of  
491 7.4%. Therefore, each aliquot of this eVLP contained an average of 1.22 $\mu$ g of GP<sub>1</sub>, or 12.2% of  
492 the total protein concentration of 10  $\mu$ g. The average  $\Delta S$  (SVG/SEE stoichiometric disparity)  
493 value was 17.3%.

494 In order to reduce the possibility of including peptide ion counts from contaminating ion  
495 species, our LC-HRMS method included a second stage MS/MS step to fragment each of the  
496 analyte peptides to confirm target sequence identity. We were able to confidently identify each  
497 of the 4 analyte quantitation peptides in at least the 3 highest dilutions of the test eVLP lot used  
498 to determine assay reproducibility. Representative MS/MS spectra of the SVG and SEE target  
499 analyst peptides are shown in **Figure 4**. With the exception of the SEE peptide at the highest  
500 dilution, peptide assignments from every dilution run were of sufficient quality to obtain non-

501 ambiguous sequence identifications. These data suggest that the newly developed LC-HRMS  
502 method can quantitate the amount of GP<sub>1</sub> in eVLP reproducibly.

503

504 **Linearity and Limit of Quantitation of Analyte Peptides**

505 To assess the limit of quantitation and sensitivity of the assay, and to ensure the range of protein  
506 concentrations tested remains linear relative to our single standard peptide concentration, we  
507 performed a linearity and limit of quantitation experiment. While the observed range of  
508 concentrations over 5 dilution points spanned from 6 to 250 fmols, it was necessary to show that  
509 we could extrapolate to concentrations that fell outside the fixed concentration represented by the  
510 AQUA peptide standards. We therefore prepared a dilution of a previously quantified eVLP lot  
511 ('A') such that a 9-point 2-fold serial dilution resulted in an on-column GP<sub>1</sub> load of between 275  
512 fmol and 1 fmol. The averaged triplicate XIC values were plotted and CV% values determined  
513 (see **Figure 1B**). The quantitation remained linear across the entire concentration range (1-  
514 275 fmol) with R<sup>2</sup> values for SVG (0.9999), SVGR (0.9972), SEE (0.9986) and IRSEE (0.9979)  
515 well within the margin of significance. While CV% values at the highest dilutions were typically  
516 less than 5%, the values in the 2 most dilute concentrations spanned a range of 7.6-17.3%. This  
517 is within acceptable limits of variability and therefore the quantitative accuracy of the assay is  
518 reliable down to 1 fmol.

519

520 **Quantitation of Multiple Lots of eVLP and Comparison with Western Blot Quantitation.**

521 The optimized protocol developed for the digestion and LC-HRMS quantitation of GP<sub>1</sub> was  
522 performed on digests of 5 different lots of eVLP's. These lots were produced by an outside  
523 contractor (Paragon BioServices, Baltimore, MD) and, at the time of our study, were being used

524 for a number of in-house animal studies. Using primary aliquots which were stored at -80 and  
525 would therefore experience only one freeze-thaw, eVLPs (10ug total protein) were digested and  
526 triplicate LC-HRMS quantitation runs were performed. Resuspension volumes for all eVLP  
527 digests were 120  $\mu$ L. As shown in **Figure 3B**, the final quantitation is derived by comparing the  
528 relative response of the 200 fmol AQUA standards (SEE and IRSEE: Set 1) to the endogenous  
529 analyte response at 4 ppm at each dilution. The average XIC's were then calculated and used in  
530 the quantitation to obtain the final concentration of GP<sub>1</sub> protein present in the eVLPs. This  
531 quantitation was performed in duplicate and the concentrations of GP<sub>1</sub> for all 5 lots of eVLPs are  
532 shown in **Table 4**. The lowest percentage of GP<sub>1</sub> relative to the total protein concentration was  
533 found in lot 'E' (1.3%) with a final concentration of 0.10 mg/ml GP<sub>1</sub>. The next lowest values are  
534 found in the lot 'D' (3.2%, 0.16 mg/ml), while the highest percentage of GP<sub>1</sub> relative to the total  
535 protein concentration was found in lot 'A' (15.8%, 0.59 mg/ml). This represents nearly an order  
536 of magnitude difference in relative GP<sub>1</sub> concentration between the VLP lots 'A' and 'E'. The  $\Delta S$   
537 values for each eVLP lot tested ranged from 7.35% for the lot 'B' to 25.5% for lot 'E'.

538 For each eVLP lot, the GP<sub>1</sub> concentration was also determined after production by the  
539 contractor via western blot with the 6D8 antibody and unpurified rGP material as a quantitation  
540 standard. As shown in **Table 4**, the range of concentration for GP<sub>1</sub> was 0.71-1.4 mg/ml. Total  
541 protein values provided for each lot ranged from 3.8-7.2 mg/ml. Since the western blot  
542 quantitation and the MS quantitation results were vastly different we decided to investigate the  
543 source of this discrepancy by repeating the western blot on the eVLP lots with the highest and  
544 lowest calculated GP<sub>1</sub> (as determined by LC-HRMS) using the 6D8 and H3D5 antibodies (see  
545 **Figure 2D and 2E**). As previously mentioned above, both eVLP lots displayed strong signals  
546 for GP<sub>1</sub> at ~140kDa using the 6D8 antibody. However, the H3D5 antibody revealed the presence

547 of truncated products previously seen in the test eVLP lot and the unpurified rGP material. These  
548 truncated products are highly abundant in the eVLP lot 'E', which returned the lowest  
549 concentration of GP<sub>1</sub> by LC-HRMS quantitation with the largest  $\Delta S$  value (25.5%), whereas  
550 eVLP lot 'A' appears to have fewer detectable GP<sub>1</sub> fragments, and returned a  $\Delta S$  value of 15.8%.

551

552 **Correlates of VLP efficacy**

553 In the hopes of using immune correlates as another measure of eVLP quality, the western blot  
554 and LC-HRMS quantitation results were compared to survival data in mice for each of these  
555 eVLP vaccine preparations. Each lot of eVLP was used to immunize mice (n=20) which were  
556 then challenged with a murine adapted Ebola Zaire virus. For each vaccination dose, volumes of  
557 eVLP were used which were surmised to contain 10 $\mu$ g of GP (as determined from the western  
558 blot quantitation performed by Paragon Biosciences). As shown in **Figure 5**, lot 'E' exhibited  
559 the lowest average survival rate after Ebola challenge (40%), and animals vaccinated with the  
560 lots 'A' and 'B' exhibited 100% survival. Lot 'E' contained the lowest calculated GP<sub>1</sub>  
561 concentration as determined by LC-HRMS whereas the lot 'A' contained the highest. The  
562 difference in survival between vaccination with lots 'A' and 'E' was significant (p=0.001). As  
563 shown in **Figure 5B**, if we plot the percent survival versus the GP<sub>1</sub> LC-HRMS quantitation in  
564 each eVLP lot, expressed as percent total protein, a strong positive correlation is observed ( $R^2 =$   
565 0.9149). A weaker correlation is observed if the absolute quantitation values for GP<sub>1</sub> (expressed  
566 as mg/ml) obtained by LC-HRMS are plotted versus survival (p=0.9025). In contrast western  
567 blot quantitation values (GP<sub>1</sub> as a percentage of total protein) did not display strong linear  
568 correlation with percent survival ( $R^2 = 0.6904$ ) and there was no correlation observed between the  
569 western blot GP1 concentrations (expressed as mg/ml) and survival.

570        The western blot quantitation of lot 'E' returned a value of 1.1mg/ml of GP<sub>1</sub>. Therefore a  
571    10 $\mu$ g GP dose would require 9.1  $\mu$ l of the eVLP preparation for vaccination. However, based on  
572    the LC-HRMS quantitation, we can retroactively estimate that the animals were given only 0.9  
573     $\mu$ g of the 10  $\mu$ g dose desired, which was adequate to protect only 4/10 animals vaccinated.  
574    Conversely, the western blot concentration for lot 'A' (1 mg/ml) is also higher than the LC-  
575    HRMS quantitation (0.59 mg/ml), and the 10 $\mu$ l dose thought to contain 10  $\mu$ g of GP<sub>1</sub> actually  
576    contained 5.9  $\mu$ g which was adequate to protect 100% of the vaccinated animals after Ebola  
577    challenge. Therefore the observed differences in eVLP efficacy between eVLP lots 'A' and 'E'  
578    are due to vastly different concentrations of antigenic GP<sub>1</sub>. From the LC-HRMS quantitation of  
579    lot 'B', which also provided 100% survival, we can calculate that a vaccine dose (based on the  
580    western blot quantitation) of 10ug GP<sub>1</sub> would actually contain 3.5  $\mu$ g which appears to be the  
581    minimal vaccination dose required to confer 100% survival in mice after Ebola challenge.

582

583 **Discussion:**

584    Provided that technical pitfalls such as incomplete protein extraction, incomplete proteolysis or  
585    protein side-chain modifications are appropriately controlled and considered, protein quantitation  
586    by MS using an AQUA strategy can be robust, accurate and reproducible, while achieving low  
587    limits of detection [28-30]. Ideally, target peptides should be well separated on a protein of  
588    interest to ensure that the entire protein is sufficiently denatured and digested prior to  
589    quantitation. Additionally, potential sites of post-translational modifications or residues  
590    susceptible to artefactual modifications should be avoided. Despite these considerations, peptide  
591    selection is an empirical exercise that balances ideal characteristics with practical limitations. For  
592    example, large proteins yield more potential target peptides than small proteins, and sequence

593 features can greatly constrain peptide selection. As we discovered with the quantitation of Ebola  
594 GP<sub>1</sub>, a protein of interest may have significant sequence homology with other proteins in a  
595 complex mixture, making it difficult to adhere to the peptide selection criteria described above.  
596 The quantitation of the Ebola Zaire GP<sub>1</sub> in eVLP preparations was a unique challenge due to the  
597 fact that during eVLP production and purification, truncated forms of the GP protein are  
598 produced and retained throughout the post-production processing. This prevented the use of  
599 target peptides located in the first 90-100 amino acids of the GP sequence. Additionally very  
600 few suitable target peptides were available in the C-terminal region of the protein due to the high  
601 frequency of glycosylation sites and high hydrophobicity. Therefore we have chosen an unusual  
602 strategy of LC-HRMS quantitation in which 2 peptides from overlapping regions of the protein  
603 are employed. This necessitated the development of a purified GP<sub>1</sub> standard to provide quality  
604 control and assay validation. The average percent accuracy of our method based on quantitation  
605 of the rGP<sub>1</sub> standard AAA analysis was 85.6%. While the HPLC fractionation we performed  
606 resulted in a significant enrichment of GP<sub>1</sub> from GP<sub>2</sub> and truncated products of GP,  
607 contaminating protein species may still be contributing to the final concentration based on AAA  
608 analysis. Indeed, the H3D5 western blot of the purified rGP<sub>1</sub> revealed immune-reactive species of  
609 lower molecular weight, which may be the source of this overestimation.

610 The presence of truncated GP products in the eVLP preparations is the likely source of  
611 variation between the quantitation of GP<sub>1</sub> with the two standard peptide sets. This hypothesis is  
612 supported by the data obtained during the rGP<sub>1</sub> standard assay development and testing. The LC-  
613 HRMS quantitation of purified, rGP<sub>1</sub> resulted in an average  $\Delta S$  of 6.6% as compared to the  
614 eVLP method validation and reproducibility trials, which showed an average  $\Delta S$  of 16.5%.  
615 Furthermore, we have never observed a higher quantitation result for GP<sub>1</sub> from peptide Set 1

616 (SEE/IRSEE) located in the middle of the molecule as compared to results obtained with peptide  
617 Set 2 (SVG/SVG.R) located near the N-terminus in any of the eVLP preparations. This supports  
618 our hypothesis that N-terminal sequence fragments in the SVG region of the protein are indeed  
619 part of the GP protein compliment of eVLP and that the moderate variability shown in our data is  
620 the result of experimental variation only.

621 Targeted MS approaches, in particular selected reaction monitoring (SRM), employing  
622 triple quadrupole mass spectrometers, have become the standard technique for quantitatively  
623 analyzing tens to hundreds of peptides and/or small molecules across a large number of samples.  
624 A limitation of triple quadrupole instruments however, is the relatively low resolution of  
625 precursor *m/z* measurements, which can allow interference from nominally isobaric background  
626 contaminants in complex mixtures. Newer instrumentation has facilitated the use of high-  
627 resolution accurate MS for quantitative analysis. This approach is often referred to as LC-HRMS  
628 and provides both qualitative and quantitative information during analysis by providing full-scan  
629 accurate mass data for the entire chromatographic run [31, 32]. Qualitative and quantitative  
630 information are obtained post-data acquisition by extracting ion chromatograms with signals  
631 centered around the *m/z* of the analyte(s) of interest and a predefined mass extraction window.  
632 This comprehensive detailed data obtained for each sample after LC-HRMS analysis was crucial  
633 for the development of a successful quantitation strategy for GP<sub>1</sub>. For example, common  
634 modifications such as deamidation cause isotopic interferences, particularly when SRM-based  
635 methods are employed using low-resolution MS [31]. Conversely, full-scan HRMS data allowed  
636 the unequivocal confirmation of deaminated endogenous target peptide species which improved  
637 the accuracy our quantitation method. Furthermore, high resolution MS/MS survey scans proved  
638 to be essential for the optimization and assessment of digestion efficiency. As revealed in this

639 work, the detailed data provided by LC-HRMS was essential to overcome the bioanalytical  
640 challenge of GP<sub>1</sub> quantitation in eVLP, and allowed us to address potential issues prior to  
641 development of a more streamlined quantitation scheme.

642 This study also highlights the superiority of mass spectrometry methods such as SRM  
643 and LC-HRMS for protein quantitation and characterization over western blotting and other  
644 immuno-affinity methods, which has been the topic of discussion in recent review articles [33,  
645 34]. A western blot assay depends on the specificity of a single antibody, and quantitative  
646 information often relies on a protein standard that may be poorly characterized, especially if  
647 evaluation is also based on reactivity to a single antibody. This can lead to quantitative  
648 inconsistencies such as those which we observed in the GP<sub>1</sub> western blot quantitation performed  
649 after eVLP production. However, there is definite value in validation by orthogonal immune-  
650 affinity approaches, and the use of the H3D5 antibody allowed us to confirm the presence of  
651 shorter versions of the GP<sub>1</sub> protein in eVLP preparations and unpurified recombinant GP  
652 material.

653 The use of crudely purified GP standard in the western blot quantitation unintentionally  
654 led to an overestimation of the final GP<sub>1</sub> concentration in eVLP, since the total protein  
655 concentration for the unpurified recombinant GP also included GP<sub>2</sub> as well as truncated protein  
656 species. Data obtained from the eVLP mouse vaccination study revealed that the amount of GP<sub>1</sub>  
657 in each eVLP lot as determined by LC-HRMS, unlike the quantitative western blot, correlated  
658 with survival after Ebola challenge. The highest observed correlation with animal survival was  
659 obtained using the percent of GP<sub>1</sub> in relation to the total protein in the VLP. This would suggest  
660 that the “density” of GP<sub>1</sub> in relation to other proteins (both viral and host derived) present in the  
661 eVLP particle is directly related to the efficacy of that particular VLP preparation.

662        The impact of the truncated products on eVLP quality or suitability for vaccination has  
663    not been determined. However, the LC-HRMS data has revealed that the eVLP lots which  
664    exhibited the lowest percent survival (lots 'D' and 'E') also contained the lowest amount of GP<sub>1</sub>  
665    and the highest  $\Delta S$  values (indicating an abundance of truncated GP<sub>1</sub> products). These truncated  
666    products may be the result of a frame-shift anomaly, ribosomal slippage or simply general  
667    protein degradation. Due to the fact that sGP is produced in greater abundance than GP<sub>1,2</sub>, and  
668    since the proteins share a common N-terminus, it is speculated that sGP functions as a decoy  
669    molecule for EBOV-specific neutralizing and non-neutralizing antibodies [35]. Additionally,  
670    recent studies have shown that sGP actively subverts the host immune response to induce cross-  
671    reactivity with epitopes it shares with membrane-bound GP<sub>1,2</sub> [36]. Therefore, truncated versions  
672    of the GP<sub>1</sub> protein may indeed compromise the quality of eVLP vaccines.

673

674    **Conclusions:**

675    A LC-HRMS approach resulted in the successful quantitation of GP<sub>1</sub> in eVLP vaccine  
676    preparations. The use of this newly developed assay will allow us to monitor variability based on  
677    GP<sub>1</sub> content, providing quality control information to further optimize and refine the eVLP  
678    production process. Finally, using this quantitative LC-HRMS approach, the total amount of GP<sub>1</sub>  
679    necessary to confer protection can be accurately determined; a crucial factor in successful  
680    vaccine development.

681

682    **List of abbreviations:**

683    EBOV (Ebola virus)

684    VLP (Virus-like particles)

685 LC-HRMS (liquid chromatography high resolution mass spectrometry)

686 GP (Glycoprotein)

687

688 **Competing interests:**

689 None of the authors have competing financial interests.

690

691 **Author's contributions:**

692 LHC participated in the experimental design, coordinated the efforts, analyzed results, and wrote  
693 the draft of the manuscript, MDW conceived the experimental design, performed sample  
694 preparation, data analysis and presentation , EB optimized instrument methods and performed all  
695 of the instrument runs, TK participated in sample processing and edited the manuscript, PD  
696 edited the manuscript, CM assisted with sample processing and assisted with the purification of  
697 the GP<sub>1</sub> standard, KOM performed the vaccinations and EBOV challenge studies in mice, and  
698 edited the manuscript. JEN conceived of the method for the purification of the GP1 standard and  
699 performed the purification procedure and edited the manuscript, TG participated in experimental  
700 design and edited the manuscript, SB contributed to study design and coordinated the research  
701 efforts. All authors have read and approved the manuscript.

702

703

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806

807 **Figure Legends:**808 **Figure 1. Target Peptide Selection and Characterization.**

809 **Panel A Top)** Sequence alignment of the 3 proteins (GP<sub>1</sub>, sGP and ssGP) derived from the Ebola  
810 GP transcript showing the locations of peptide candidates for use in the quantification of Ebola  
811 GP<sub>1</sub> (red dotted boxes) as well as the location of peptides rejected for the final assay (black  
812 boxes). All three proteins share sequence homology in the first 295 amino acids. Peptides  
813 identified in survey runs were evaluated for absence of post translational modifications,  
814 ionization efficiency and protein location.. **Panel A Bottom)** Schematic of fully processed GP<sub>1,2</sub>  
815 transmembrane protein, showing the location of the receptor binding site (RBS) and mucin-like  
816 domain (MLD) of GP<sub>1</sub>, as well as the extracellular domain (ECD), transmembrane region (TM)  
817 and cytoplasmic tail (CT) of GP<sub>2</sub>. GP<sub>1</sub> and GP<sub>2</sub> are disulfide linked to form the mature GP<sub>1,2</sub>  
818 complex. **Panel B.** Standard curves for each target analyte peptide over a 9 point dilution

819 showing linearity from 275 fmols to 1 fmol total GP<sub>1</sub>. An aliquot of the previously quantified  
820 eVLP lot 'A' (200 fmols/µL SEE at 120 µL dilution) was resuspended 83 µL 40% acetonitrile,  
821 0.1% Formic (137.5 fmols/µL) and serially diluted. A 2 µL injection utilizing the described  
822 instrument method was run in triplicate for each dilution. R<sup>2</sup> values for all four peptides are well  
823 within the margin of significance for linearity. Also shown in tabular form are the %CV values  
824 for each triplicate XIC measurement for each peptide at each dilution. These data indicate  
825 linearity down to 1 fmol with the largest CV% (SVGR – 17.3%) in dilution number '8' of the  
826 serially diluted series. **Panel C.** AQUA-SVG peptide signal response for non-deamidated  
827 (circle) and deamidated (triangle) peptide. AQUA-SVG peptide was deamidated by incubating  
828 40 pmols at 50°C/pH 8.0 for 2.5 days while a matching 40 pmol aliquot was stored at -20°C. A  
829 5-point, 2-fold serial dilution was performed resulting in a 250 to 15.6 fmol/µL concentration  
830 range for each sample. LC-HRMS was run in triplicate on each dilution and the average counts  
831 plotted.

832

833 **Figure 2. Purification and characterization of a recombinant GP<sub>1</sub> standard.**

834 **Panel A.** Representative chromatogram of preparative C4 reverse phase HPLC of 300µg  
835 reduced recombinant GP material indicating fraction collection points. **Panel B.** SDS PAGE  
836 followed by silver-staining of fractions 1-7 showing the separation of GP<sub>1</sub> (top arrow) and GP<sub>2</sub>  
837 (bottom arrow). Material from fraction 1 was divided into 1.8µg aliquots and used for  
838 quantitation standard. **Panel C.** Silver stained SDS PAGE performed under reducing conditions  
839 comparing the rGP starting material and the purified rGP<sub>1</sub> standard. GP1 (top arrow) and GP2  
840 (bottom arrow). **Panel D.** Western blot of eVLP Lot 'A', eVLP Lot 'E', unpurified rGP and the  
841 purified rGP<sub>1</sub> standard using the monoclonal antibody 6D8 showing the detection of fully

842 glycosylated GP<sub>1</sub> (arrow). **Panel E.** Western blot of eVLP Lot 'A', eVLP Lot 'E', unpurified rGP  
843 and the purified rGP<sub>1</sub> standard using the monoclonal antibody H3D5 showing the detection of  
844 fully glycosylated GP<sub>1</sub> (arrow) and GP protein fragments.

845

846 **Figure 3. Illustration of the eVLP quantitation method workflow, calculations and eVLP**  
847 **GP<sub>1</sub> result table.**

848 **Panel A.** VLP digests are resuspended in 120  $\mu$ L and five 2-fold serial dilutions performed.  
849 Each dilution is mixed 1:1 with a solution containing 200 fmol/ $\mu$ L of each of the four  
850 isotopically labeled AQUA peptides and run in triplicate using 2  $\mu$ L injections. **Panel B.**  
851 Method used for calculating the GP<sub>1</sub> concentration in the rGP<sub>1</sub> standard at each dilution.  
852 Average XIC area counts from the 2+ and 3+ charge states are first summed for each AQUA  
853 ( $\mathbf{P}_{\text{aq}}$ ) and analyte ( $\mathbf{P}_{\text{v}}$ ) peptide. The values from the SEE and IRSEE are summed to provide the  
854 total counts for Peptide 'Set 1' and the SVG and SVGR values are summed to provide total  
855 counts for Peptide 'Set 2'. The final quantitation is derived by comparing the relative response  
856 of the 200 fmol AQUA standard to the endogenous analyte response at 4 ppm and averaging the  
857 response between the 2 peptide sets. For absolute eVLP rGP<sub>1</sub> quantitation, only the values  
858 derived from peptide set 1 (SEE/IRSEE) were used.

859

860 **Figure 4. Average MS/MS fragmentation spectra of the SVG and SEE peptide.**

861 Replicate CID fragmentation spectra and 300 ppm theoretical ion tables of the SVG (**A,B**) and  
862 SEE (**C,D**) analyte peptides derived from eVLP lot 'A'. Panels A and B represent the y-series  
863 assignments at the 1:2 (1) and 1:32 (5) dilution samples respectively (see dilution scheme in

864 Figure 3A). Prominent y-series sequence ions are indicated. The SVG series contains 10  
865 consecutive y-series ions resulting in a MASCOT Ions Score of 69 and an Expect score of  
866  $6.7 \times 10^{-6}$  at dilution '1' with the '5' dilution Ions Score at 57 with an Expect Score of  $8.8 \times 10^{-5}$ .  
867 The SEE peptide contains 7 y-series ions in both the '1' and '5' dilutions with Ions Scores of 55  
868 and 41 with Expect Scores of  $8.2 \times 10^{-5}$  and 0.0021 respectively. With the exception of the SEE  
869 '5' dilution, peptide assignments from every dilution run were of sufficient quality to obtain non-  
870 ambiguous sequence identifications.

871

872 **Figure 5. Survival data in mice for quantified eVLP lots and correlation with LC-HRMS**  
873 **and western blot quantitation. A)** Average percent survival (grey bars) after two vaccinations  
874 in mice (n=10 for each eVLP lot) with 10 $\mu$ g of GP<sub>1</sub> (as calculated from 6D8 WB results) of the  
875 indicated lot. Vaccinations were three weeks apart, with four weeks between the final  
876 vaccination and the challenge. The percent GP<sub>1</sub> to total protein concentration (right axis)  
877 obtained using LC-HRMS is represented by the white bars. Survival in all vaccinated groups was  
878 significant (p<.005) when compared to saline controls. Fisher's Exact test was used to compare  
879 survival between the group vaccinated with eVLP lot 'E' and the other lots (\* indicates p < 0.01,  
880 \*\* indicates p < .001) **B)** LC-HRMS and western plot values for GP<sub>1</sub> content in each eVLP lot  
881 tested (based on % total protein or mg/ml) were plotted against percent survival in mice after  
882 EBOV challenge (since Lots A and B both gave 100% the lower lot B value was used resulting  
883 in a total of 4 datapoints). The strongest linear correlation ( $R^2=0.9149$ ) was obtained with the  
884 LC-HRMS GP<sub>1</sub> values based on % total protein followed by the LC-HRMS values for GP<sub>1</sub> in  
885 mg/ml ( $R^2=0.9025$ ).

886

887 **Supplemental Figure 1. XIC profiles of each peptide charge state used in the quantitation**  
888 **showing the retention time alignment at 4 ppm.** Data was taken from eVLP lot 'B' dilution  
889 '2' replicate. The 4 profiles from each peptide are ordered from AQUA 2+ (H 2+), analyte 2+ (L  
890 2+), AQUA 3+ (H 3+) and analyte 3+ (L 3+). **Panel A** represents the SVG and SVGR peptide  
891 Set 2, **Panel B** is the SEE and IRSEE peptide Set 1 and **Panel C** shows the XIC profiles of the  
892 deamidated SVG and SVGR analyte peptides. XIC values were acquired with at least 7 data  
893 points sampled across the elution profile.

894

895 **Supplemental Figure 2. The binary behavior of the 2 asparagines (N) within the SVG**  
896 **peptide.** In order to assess whether the AQUA SVG peptide counts would show a similar  
897 response to the deamidated analyte SVG peptide a 40 pmol aliquot of the AQUA peptide SVG  
898 standard was incubated at 55°C for 2.5 days at pH 8.1 resulting in complete deamidation likely at  
899 asparagine 9. Target m/z values were 969.4969 (**A**), 646.6684 (**B**), 969.0069 (**C**), 646.3404 (**D**),  
900 969.9909 (**E**) and 646.9964 (**F**). The most abundant species by far is the singly deamidated 2+  
901 ion followed by the singly deamidated 3+ ion. The doubly deamidated ions comprise 5% of the  
902 total counts. \*Note the complete absence of signal in the non-deamidated mass ranges.

903

904 **Supplemental Figure 3. LC-MS/MS protein identification of coomassie blue stained bands**  
905 (**A**) derived from a 5 µg aliquot of the eVLP lot 'B' after SDS-PAGE analysis. The 10 highest  
906 intensity bands from a coomassie stained 4-12% NuPAGE Novex Bis-Tris gel were excised and  
907 digested with Trypsin/Lys-C. Each sample was analyzed on a 60 minute LC-MS/MS survey MS  
908 run and searched with MASCOT (v. 2.4) against an SProt (2014\_02) database with human and  
909 *Ebola zaire* taxonomy specified. Locations of the 5 most prominent peptides are indicated as

910 well as the total number of GP<sub>1</sub> peptides observed for each sample (GP<sub>1</sub> peptide counts). A  
 911 minimum Expect score of 0.005 with an FDR of 1% was used for peptide validation. No N-  
 912 terminal peptides downstream of AA 192 were observed in gel bands 7 thru 10. These data  
 913 indicate that while the majority of the GP<sub>1</sub> protein appears to be full-length, a significant number  
 914 of N-terminal GP<sub>1</sub> fragments are present. **(B)**An identical sample was analyzed via western blot  
 915 using the H3D5 antibody.

916

**Table 1.** Masses of analyte and AQUA standard peptides used for quantitation of GP<sub>1</sub> in eVLP

		Analyte		AQUA Standard	
Set	Sequence	2(+) m/z	3(+) m/z	2(+) m/z	3(+) m/z
1	SEELSFTAVSNR	670.3281	447.2211	675.3322	450.5572
1	IRSEELSFTAVSNR	804.9206	536.9495	809.9248	540.2856
2	SVGLNLEGNGVATDVPSATK	964.9998	643.669	969.0069	646.3404
2	SVGLNLEGNGVATDVPSATKR	1043.0498	695.7027	1048.0549	699.0388
2	SVGLNLEGNGVATDVPSATK-deam	965.4918	643.997	N/A	N/A
2	SVGLNLEGNGVATDVPSATKR-deam	1043.5424	696.0307	N/A	N/A

917

918

**Table 2.** HR/AM-MS method validation using purified recombinant GP<sub>1</sub> standard.

Trial	Dilution	Peptide	On-Column		Total In Sample (μg)	Ave. (μg)	ΔS	Accuracy (%)	Precision (% CV)
			fmoles	ng					
1	60	Set 1	472.1	25.9	1.55	1.50	-7.00%	83.2	0.35
		Set 2	439.0	24.0	1.44				
2	60	Set 1	432.3	32.2	1.42	1.49	9.30%	82.6	3.2
		Set 2	472.4	35.2	1.55				
3	80	Set 1	347.7	25.9	1.52	1.56	4.60%	86.6	3.2
		Set 2	363.7	27.1	1.59				
4	80	Set 1	363.3	27.0	1.59	1.64	5.50%	90.1	3.2
		Set 2	383.4	28.5	1.68				

919

920

921

**Table 3.** GP<sub>1</sub> quantitation in three replicates of a single eVLP lot

Sample	Digest ( $\mu$ g)	fmoles		GP <sub>1</sub>				
		Pep Set 1	Pep Set 2	Aliquot ( $\mu$ g)	Concentration (mg/ml)	Pct. Total Protein	$\Delta S$ (%)	CV%
Lot A (1)	10	199.1	231.1	1.31	0.62	13.1	16.1	7.40%
Lot A (2)	10	172.3	207.1	1.13	0.53	11.3	20.2	
Lot A (3)	10	183.8	212.2	1.21	0.61	12.1	15.5	

922

923

**Table 4.** LC-HRMS results of GP<sub>1</sub> quantitation in 5 lots of eVLP and comparison with quantitative western blot values

Lot	Concentration (mg/ml)			MS (CV%)	Average $\Delta S$ (%)	% TP
	Protein	rGP WB	MS			
A	4.7	1.0	0.59	5.6%	15.8	12.6
B	3.8	1.14	0.35	10.3%	7.35	9.2
C	4.8	1.4	0.31	11.6%	12.7	6.5
D	4.9	0.7	0.16	8.8%	24.1	3.2
E	7.2	1.1	0.10	5.4%	25.5	1.3

924

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Figure 1 DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

A

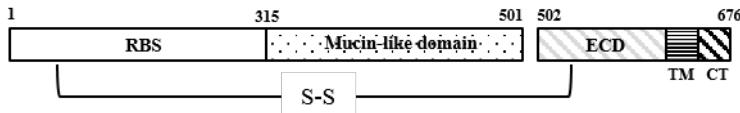
1 MGVTGILQLPDRFRKRTSFLWVILFQRTFSIPLGVIH<sup>1</sup>STLQVSDVDKLV<sup>2</sup>RDKLSST  
 1 MGVTGILQLPDRFRKRTSFLWVILFQRTFSIPLGVIH<sup>1</sup>STLQVSDVDKLV<sup>2</sup>RDKLSST  
 1 MGVTGILQLPDRFRKRTSFLWVILFQRTFSIPLGVIH<sup>1</sup>STLQVSDVDKLV<sup>2</sup>RDKLSST  
 \*\*\*\*\*  
 61 NQLR<sup>3</sup>SVGLNLEGNGVATDVP<sup>4</sup>SATKR<sup>5</sup>NGFRSGVPPKVNNYEAGEWAEN<sup>6</sup>CY<sup>7</sup>NLEI<sup>8</sup>KPDGSE  
 61 NQLR<sup>3</sup>SVGLNLEGNGVATDVP<sup>4</sup>SATKR<sup>5</sup>NGFRSGVPPKVNNYEAGEWAEN<sup>6</sup>CY<sup>7</sup>NLEI<sup>8</sup>KPDGSE  
 61 NQLR<sup>3</sup>SVGLNLEGNGVATDVP<sup>4</sup>SATKR<sup>5</sup>NGFRSGVPPKVNNYEAGEWAEN<sup>6</sup>CY<sup>7</sup>NLEI<sup>8</sup>KPDGSE  
 \*\*\*\*\*  
 121 LPAAPDG<sup>1</sup>RG<sup>2</sup>FPR<sup>3</sup>RYVHKVSGTGP<sup>4</sup>AGDFAFHKEGAF<sup>5</sup>FLYDRLASTV<sup>6</sup>Y<sup>7</sup>GTTFAEGV  
 121 LPAAPDG<sup>1</sup>RG<sup>2</sup>FPR<sup>3</sup>RYVHKVSGTGP<sup>4</sup>AGDFAFHKEGAF<sup>5</sup>FLYDRLASTV<sup>6</sup>Y<sup>7</sup>GTTFAEGV  
 121 LPAAPDG<sup>1</sup>RG<sup>2</sup>FPR<sup>3</sup>RYVHKVSGTGP<sup>4</sup>AGDFAFHKEGAF<sup>5</sup>FLYDRLASTV<sup>6</sup>Y<sup>7</sup>GTTFAEGV  
 \*\*\*\*\*  
 181 VAFLILPQAK<sup>1</sup>KDF<sup>2</sup>SSHPLREP<sup>3</sup>V<sup>4</sup>ATEDPSSGYY<sup>5</sup>TTT<sup>6</sup>IRYQATGFGT<sup>7</sup>N<sup>8</sup>ETEYLFEVD<sup>9</sup>NLT  
 181 VAFLILPQAK<sup>1</sup>KDF<sup>2</sup>SSHPLREP<sup>3</sup>V<sup>4</sup>ATEDPSSGYY<sup>5</sup>TTT<sup>6</sup>IRYQATGFGT<sup>7</sup>N<sup>8</sup>ETEYLFEVD<sup>9</sup>NLT  
 181 VAFLILPQAK<sup>1</sup>KDF<sup>2</sup>SSHPLREP<sup>3</sup>V<sup>4</sup>ATEDPSSGYY<sup>5</sup>TTT<sup>6</sup>IRYQATGFGT<sup>7</sup>N<sup>8</sup>ETEYLFEVD<sup>9</sup>NLT  
 \*\*\*\*\*  
 241 YVQLESRFTPQFLQL<sup>1</sup>NETIY<sup>2</sup>TSGKRS<sup>3</sup>TTGK<sup>4</sup>L<sup>5</sup>W<sup>6</sup>KVN<sup>7</sup>PEID<sup>8</sup>TT<sup>9</sup>GEWA<sup>10</sup>F<sup>11</sup>WETK<sup>12</sup>K<sup>13</sup>TRK  
 241 YVQLESRFTPQFLQL<sup>1</sup>NETIY<sup>2</sup>TSGKRS<sup>3</sup>TTGK<sup>4</sup>L<sup>5</sup>W<sup>6</sup>KVN<sup>7</sup>PEID<sup>8</sup>TT<sup>9</sup>GEWA<sup>10</sup>F<sup>11</sup>WETK<sup>12</sup>K<sup>13</sup>TSLEK  
 241 YVQLESRFTPQFLQL<sup>1</sup>NETIY<sup>2</sup>TSGKRS<sup>3</sup>TTGK<sup>4</sup>L<sup>5</sup>W<sup>6</sup>KVN<sup>7</sup>PEID<sup>8</sup>TT<sup>9</sup>GEWA<sup>10</sup>F<sup>11</sup>WETK<sup>12</sup>K<sup>13</sup>PPH--  
 \*\*\*\*\*  
 301 IRSEELSF<sup>1</sup>TAV<sup>2</sup>-----<sup>3</sup>SNR<sup>4</sup>AK<sup>5</sup>ISQSPARTSSD<sup>6</sup>PGT<sup>7</sup>TTTEDHKIMASE<sup>8</sup>SSAMV  
 301 FAVK<sup>9</sup>SC<sup>10</sup>SOLY<sup>11</sup>TEPKTSVVR<sup>12</sup>RELLPTQ<sup>13</sup>GPTQQLKTTK<sup>14</sup>S<sup>15</sup>---WLQKIP<sup>16</sup>---QW-  
 299 -----  
 352 QVHSQGREAAVSHLTLAT<sup>1</sup>STSPQ<sup>2</sup>PTTKPGPD<sup>3</sup>I<sup>4</sup>SHNTPVY<sup>5</sup>KLD<sup>6</sup>SEATQ<sup>7</sup>VEQHRR<sup>8</sup>T  
 351 -FKCTV<sup>9</sup>KEGKLQ<sup>10</sup>---CRI<sup>11</sup>-----  
 299 -----  
 412 D<sup>1</sup>ND<sup>2</sup>STASD<sup>3</sup>TPPATTAA<sup>4</sup>GPLKA<sup>5</sup>ENT<sup>6</sup>TSKG<sup>7</sup>D<sup>8</sup>LLD<sup>9</sup>PA<sup>10</sup>TT<sup>11</sup>SPQ<sup>12</sup>N<sup>13</sup>HSETAGN<sup>14</sup>I<sup>15</sup>NTHQDTGE  
 365 -----  
 299 -----  
 472 ESASSG<sup>1</sup>I<sup>2</sup>LG<sup>3</sup>LITNTI<sup>4</sup>AGVAGL<sup>5</sup>ITG<sup>6</sup>GR<sup>7</sup>ARR<sup>8</sup>  
 365 -----  
 299 -----

Signal Peptide  
 Disulfide

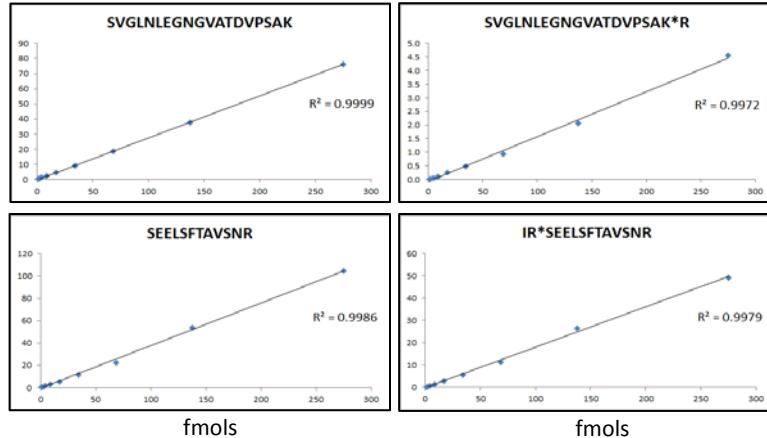
Glycosylation

GP1

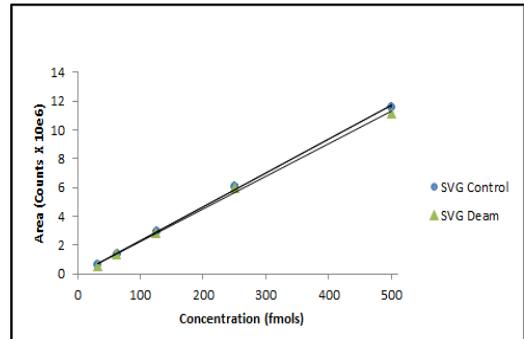
GP2



B

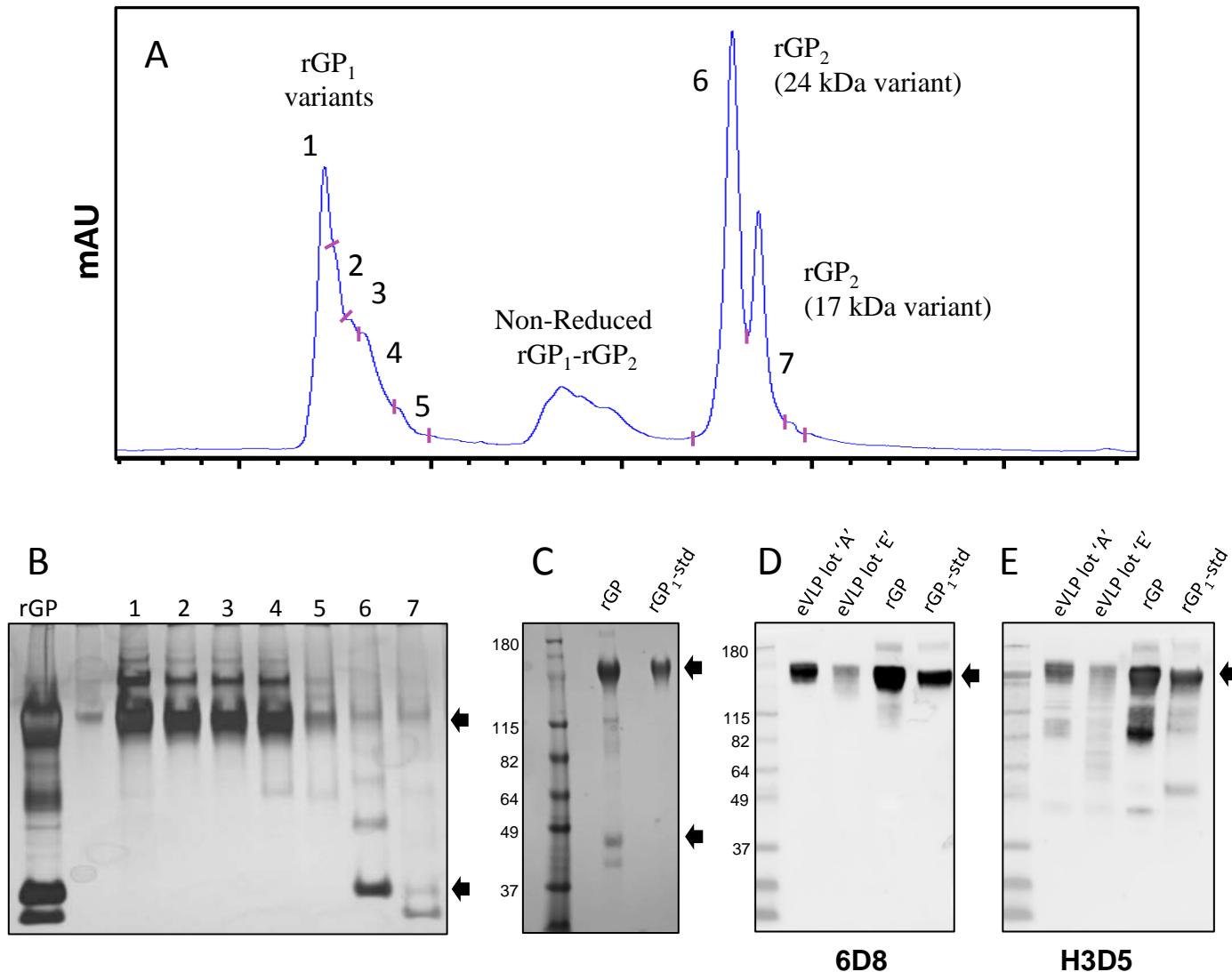


C



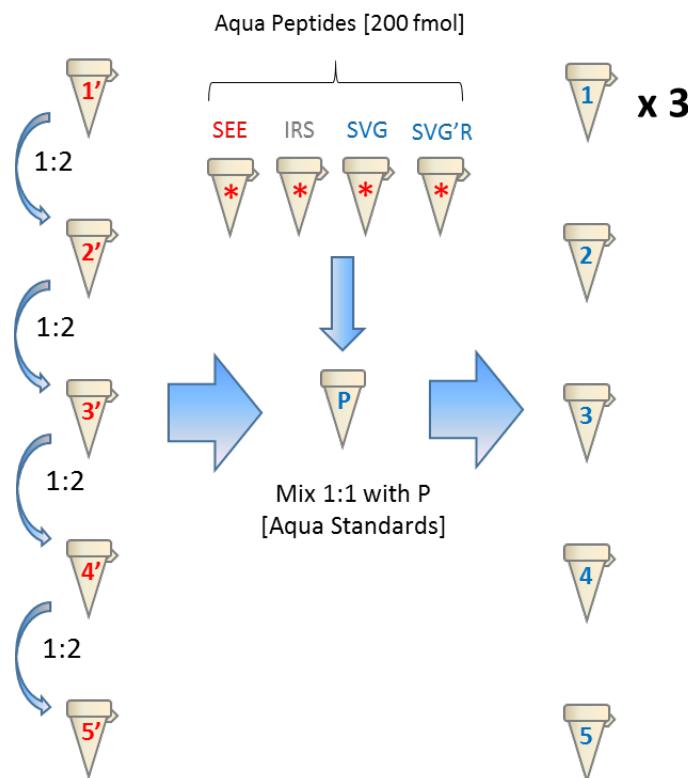
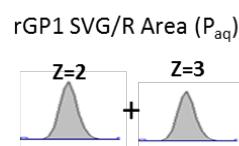
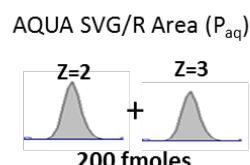
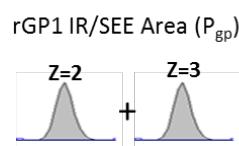
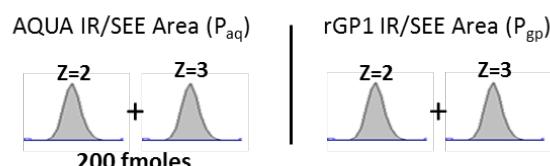
Peptide	Dilution (CV%)								
	1	2	3	4	5	6	7	8	9
SVG	3.9	2.3	5.1	3.0	2.8	12.9	2.8	10.8	11.9
SVGR	4.3	5.1	7.4	5.1	8.7	7.6	1.0	17.3	16.8
SEE	5.6	4.9	8.1	4.2	4.8	11.2	5.9	7.6	14.9
IRSEE	3.7	5.6	1.8	6.1	6.1	5.4	11.4	8.4	16.2

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Figure 3

Workflow**B** Absolute rGP<sub>1</sub> Concentration

Average (n=3)

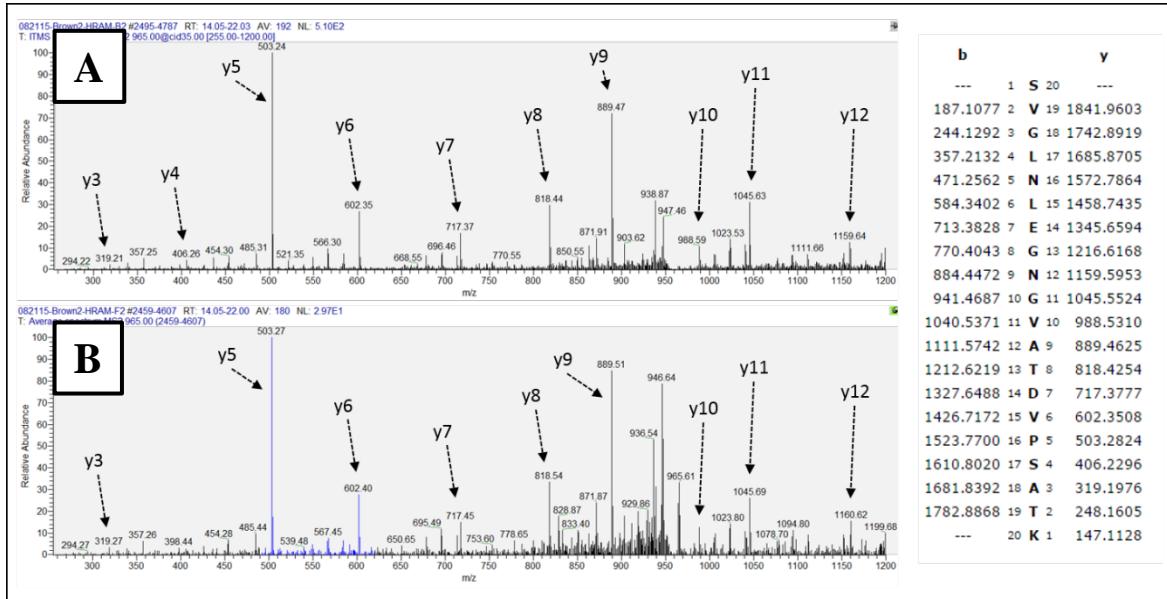
$$\left( 200 \text{ fmol} \times \frac{P_{v\text{SEE}}}{P_{aq\text{SEE}}} \right) + \left( 200 \text{ fmol} \times \frac{P_{v\text{IRSEE}}}{P_{aq\text{IRSEE}}} \right) = \text{fmol IR/SEE on column}$$

$$\left( 200 \text{ fmol} \times \frac{P_{v\text{SVG}}}{P_{aq\text{SVG}}} \right) + \left( 200 \text{ fmol} \times \frac{P_{v\text{SVGR}}}{P_{aq\text{SVGR}}} \right) = \text{fmol SVG/R on column}$$

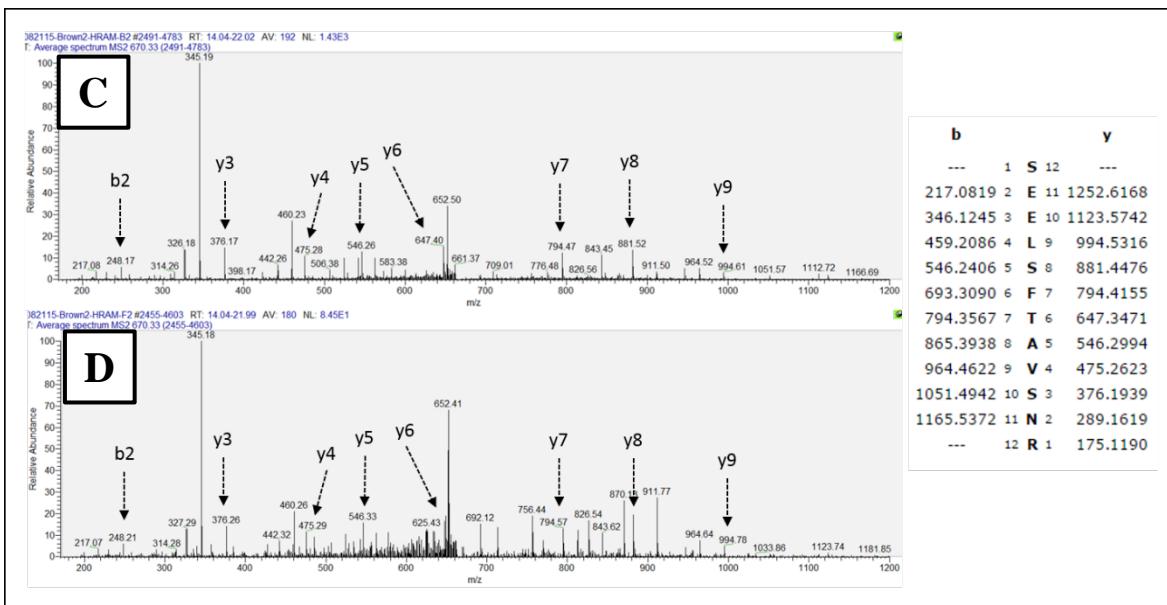
AVERAGE  $\left( \begin{array}{c} \text{fmol IR/SEE} \\ + \\ \text{UNDILUTED} \end{array} \right) = \text{fmol GP}_1 \text{ on column}$

Figure 4 DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

## SVGLNLEGNGVATDVPSATK



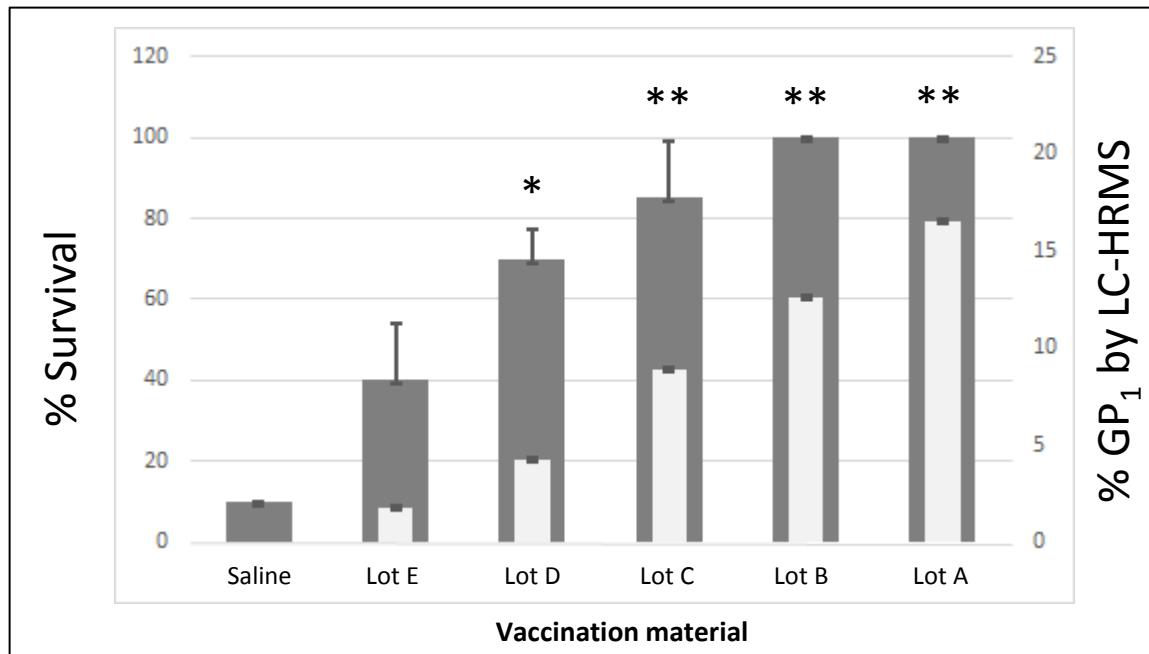
## SEELSFTAVSNR



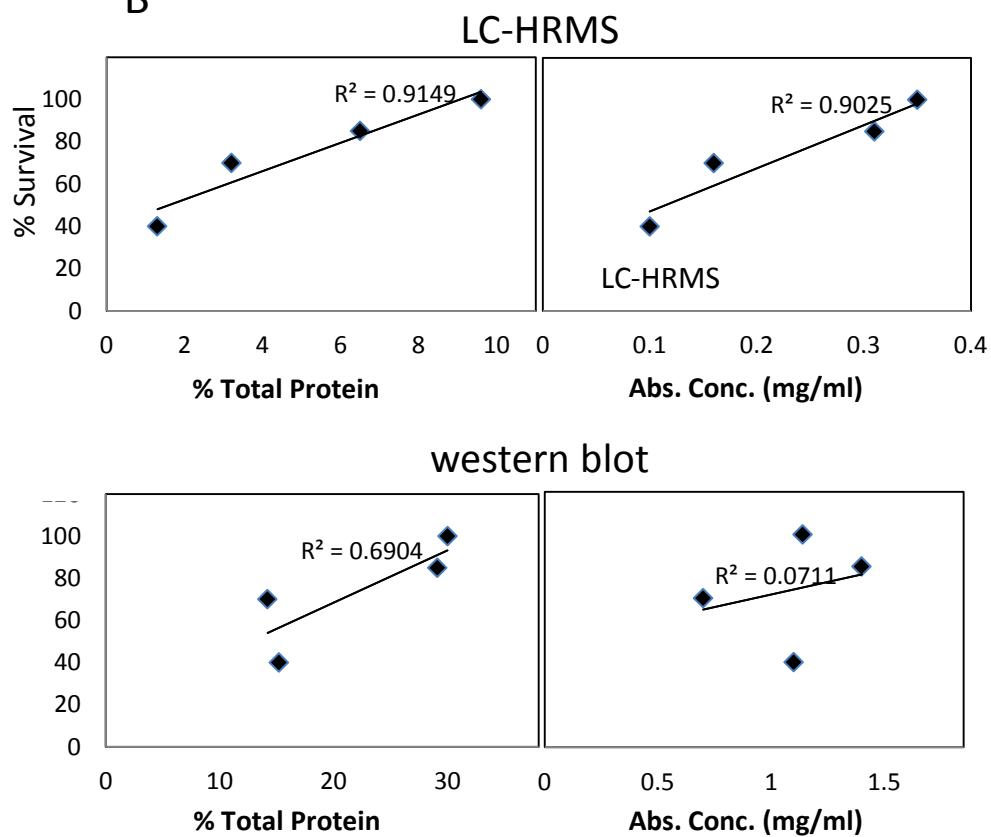
UNCLASSIFIED

Figure 5 DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.

A



B



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